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UPTAKE, DISTRIBUTION IN DIFFERENT TISSUES, AND GENOTOXICITY OF IMIDACLOPRID IN THE FRESHWATER FISH *AUSTRALOHEROS FACETUS*

FERNANDO G. ITURBURU,† MARKUS ZÖMISCH,‡ ANA M. PANZERI,† ANDREA C. CRUPKIN,§
VALESKA CONTARDO-JARA,‡ STEPHAN PFLUGMACHER,‡ and MIRTA L. MENONE*†
†Laboratorio de Ecotoxicología, Instituto de Investigaciones Marinas y Costeras (IIMYC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Mar del Plata, Buenos Aires, Argentina
‡Ecological Impact Research and Ecotoxicology, Institute of Ecology, Technische Universität Berlin, Berlin, Germany
§Laboratorio de Ecotoxicología, Universidad Nacional de Mar del Plata, Buenos Aires, Argentina

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Abstract: The neonicotinoid imidacloprid is under re-evaluation by regulatory agencies because of the poor current information available regarding its potential effects. One of the goals of the present study was to determine imidacloprid uptake and distribution in the freshwater fish *Australoheros facetus* experimentally exposed for 24 h and 48 h to 100 μg/L, 300 μg/L, and 2500 μg/L. The toxicity of imidacloprid to fish reported in the literature is in the milligrams per liter or gram per liter range, but sublethal effects at micrograms per liter in some groups other than fish have been described. Another goal of the present study was to evaluate imidacloprid's potential genotoxicity and to compare it between the individual compound and a commercial formulation. Concentrations of imidacloprid were measured in water, brain, muscle, gills, gut, liver, and blood by liquid chromatography-tandem mass spectrometry. Imidacloprid was detected in all the tissues tested. Concentrations were higher after 48 h than after 24 h in liver, gills, gut, and muscle, whereas in brain and blood they were similar at both exposure times. Although there was no accumulation, only uptake, of imidacloprid, genotoxicity was observed. In fish exposed to IMIDA NOVA 35®, increased micronucleus frequency at 100 μg/L and 1000 μg/L was detected, whereas in the imidacloprid active ingredient bioassay it increased only at 1000 μg/L imidacloprid. The present findings warn of the possible consequences that fish living in freshwater ecosystems can suffer. *Environ Toxicol Chem* 2016;9999:1–10. © 2016 SETAC

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INTRODUCTION

Since their introduction in 1991, neonicotinoid insecticides have been taking over the market [1]. At the time of writing, they are registered in more than 120 countries [2]. They are generally polar, nonvolatile, and hydrolytically stable [3]. Despite their advantages, like safeness to pesticide operators, unique mode of action, and selective efficacy, the European Commission has adopted a proposal [4] to restrict the use of 3 neonicotinoid pesticides including imidacloprid for a period of 2 yr (from 2013 to 2015). This decision was based on the European Food Safety Authority report, which included a risk assessment of imidacloprid for bees [5].

At the time of writing, there is a controversy in the scientific world about the role of neonicotinoids in colony collapse disorder [6,7] and other possible effects on pollinators (for a complete review, see van der Sluijs et al. [8]). In addition, in Canada in February 2014 there were 87 registration records listed on the Pest Management Regulatory Agency label registry for products containing the main neonicotinoid insecticides imidacloprid, clothianidin, or thiamethoxam. They and their associated products are currently under re-evaluation by the Health Canada Pest Management Regulatory Agency [9].

Imidacloprid was introduced to the agrochemical market in 1991 by Bayer, and it was the first neonicotinoid available for use. In agriculture it is applied to treat seeds, soil, and plants [10], constituting one of the most sold insecticides in the world [2,11]. Imidacloprid has selective toxicity for insects but not vertebrates [12]. In vitro studies have demonstrated that the electrostatic interactions of the nitroimine group and bridgehead nitrogen in imidacloprid (with particular amino acid residues of nicotinic acetylcholine receptors) are likely to have key roles in determining the selective toxicity of this insecticide [12]. However, the neurotoxicity of neonicotinoids is well established for a wide range of organisms [13]. Imidacloprid is highly soluble in water, and therefore, its lipophobicity suggests that it would not be accumulated in the organisms.

The potential for leaching into surface waters is 1 of the major concerns surrounding extensive use of neonicotinoids on agricultural fields, especially those in close proximity to water bodies. In fact, the presence of neonicotinoids in runoff from soils or in groundwater as a result of leaching has been demonstrated [14]. Therefore, its current massive use worldwide makes its study relevant from an ecotoxicological point of view.

Although little monitoring data are available regarding the concentrations of these insecticides in surface water, their occurrence has been reported in different water bodies around the world. In The Netherlands environmental concentrations ranged from 1.1 μg/L to 320 μg/L [15,16]. In Spain Masiá et al. [17] reported concentrations in the range 0.002 μg/L to 0.02 μg/L. Imidacloprid has been detected at concentrations of >0.1 μg/L in 2 of 57 samples from New Brunswick, Canada [9]. On the other hand, samples from 77 groundwater wells were collected in 2008 and 2009 from potato-producing regions in Quebec Province, Canada. Imidacloprid was detected in 61% of the wells including farms and neighboring properties at concentrations of up to 6.1 μg/L [18]. In the United States imidacloprid has been detected in 89% of samples tested; concentrations exceeded the US Environmental Protection

* Address correspondence to mirta.menone@gmail.com
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Agency's chronic invertebrate aquatic life benchmark of 1.05 mg/L in 19% of samples; 71% of them exceeded also the Canadian interim water quality guideline for freshwater aquatic life, the maximum imidacloprid concentration detected being 3.29 μg/L [19]. Vietnam surface waters showed imidacloprid concentrations ranging 0.12 μg/L to 0.19 μg/L [20]; in Australia the range has been 0.04 μg/L to 4.56 μg/L [21]. Hence, there are enough data to establish that imidacloprid reaches rivers and creeks near applications to agricultural fields and groundwater all over the world. Imidacloprid was analyzed in surface waters from 4 sub-basins in Argentina, but only traces not quantified were detected [22].

Fish accumulate chemicals both by ingestion of contaminated food and by contact of their gill surfaces and skin with contaminated water. In general, an accumulated chemical is distributed throughout the fish and reaches a site of action to cause toxic effects [23]. Fish play an important ecological role in food chains by their function of transferring energy from lower trophic levels to higher ones, and they are used as bioindicators to monitor pollution of aquatic systems [24]. The freshwater fish *Australoheros facetus* is a cichlid that inhabits the Paraná, Uruguay, and de la Plata basins [25]. This native species is easy to rear and breed under laboratory conditions, and it has been used in metal and pesticide toxicology studies [26,27].

Few reports concerning imidacloprid toxicity to fish are available in the literature. In general, reported 96-h 50% lethal concentration (LC50) and 7-d or 14-d lowest-observed-effect concentration values in the literature are in the milligrams per liter or grams per liter range with similar mortality-inducing concentrations in several species [9]. In fish, no toxicity of analytical grade imidacloprid to development of zebrafish embryos was observed even at 320 mg/L, although the survival of adult zebrafish (*Danio rerio*) exposed to the commercial formulation Confidor SL 200 was more affected than embryo development [10]. In flounder (*Paralichthys olivaceus*), in vitro cytotoxicity median inhibitory concentrations of 38.5 μg/L to 41.9 μg/L of technical imidacloprid were reported [28], whereas other species like rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) presented 96-h LC50 values of 211 mg/L and 280 mg/L, respectively [10]. Other negative effects, like the alteration of the neurobehavioral function (e.g., decreased swimming activity) in early-life and adult zebrafish [29] or the stress syndrome in juvenile medaka fish (*Oryzias latipes*) [30], have been described. Other vertebrates also suffer imidacloprid effects. For example, increased DNA damage in human peripheral blood lymphocytes exposed in vitro to 20 μM imidacloprid (~5 mg/L) [31] have been reported.

There are good reasons to believe that wildlife and human populations are increasingly affected by mutation pressures resulting from environmental contamination. Chemical contamination can cause population reduction through somatic and heritable mutations, as well as nongenetic modes of toxicity [32]. Field studies are accumulating evidence of the negative effects of different types of pollutants on the survival and reproduction of wildlife. Recently, a feral population of nase fish (*Chondrostoma nasus*) was studied in the Rhone River watershed (France), and the authors found that mortality and abnormality rates, measured at both hatching and the end of yolk sac resorption stages, followed the same trend as the sperm DNA damage, demonstrating an impact of river water quality on fish fitness through a loss of sperm DNA integrity [33]. The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis and in other effects such as reproductive impairment. The micronucleus assay has emerged as 1 of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably [34]. No data on the potential genotoxicity of imidacloprid to fish are available to date in the public literature, but other taxa have been studied. Genotoxic effects measured through micronucleus and "comet" assay have been reported in amphibians. Increased frequency of micronucleus was observed at 8 mg/L and 32 mg/L imidacloprid in tadpoles of *Rana* N. Hallowell exposed for 7 d [35] and at 15 mg/L and 30 mg/L imidacloprid in Montevideo tree frog (*Hypsiboas pulchellus*) tadpoles exposed for 48 h [36]. An increase in the genetic damage index was observed in tadpoles of *H. pulchellus* treated with 30 mg/L imidacloprid for 48 h [36], whereas the index increased at 2.5 mg/L to 37.5 mg/L concentration range when tadpoles were exposed to the commercial formulation Glacoxan Imida (Punch Química SA) [37].

Considering that there are no available data on imidacloprid uptake, distribution, and accumulation in fish or other poikilotherm taxa or genotoxicity in fish species, the goals of the present study were 1) to determine imidacloprid uptake and distribution in different tissues of the freshwater fish *A. facetus* experimentally exposed for 24 h and 48 h, 2) to evaluate potential genotoxic effects, and 3) to compare the genotoxicity between the individual compound and a commercial formulation.

MATERIALS AND METHODS

Test animals

Adult *A. facetus* were collected in nonanthropized freshwater bodies around Mar del Plata city (Buenos Aires Province, Argentina, 37°53′S, 57°59′W) and acclimatized for 2 mo to laboratory conditions in 140-L tanks. The photoperiod was set at 12:12-h light:dark. The temperature of the tap water in the tanks was maintained at 15 ± 1 °C, and the pH was 8.5.

Chemicals

Imidacloprid (*N*-{1-[(6-chloro-3-pyridyl) methyl]-4,5-dihydroimidazol-2-yl} nitramide) analytical standard was purchased from Sigma-Aldrich (CAS no. 138261-41-3). Environmentally relevant physical–chemical properties of imidacloprid are a water solubility of 610 mg/L at 20 °C and an octanol–water partitioning coefficient (log K_{OW}) of 0.57 [38].

The commercial formulation IMIDA NOVA 35® (NOVA) 35% concentrated liquid was used.

Other chemicals used for chromatography (acetonitrile) were of mass spectrometry grade (HiPerSolv CHROMANORM), and formic acid was agricultural grade (EMSURE, purchased from Merck). Methanol used for extractions was of 99% purity.

A stock solution was prepared by diluting imidacloprid in dimethyl sulfoxide (DMSO). The appropriate exposure medium was prepared by diluting different amounts of the stock solution in tap water immediately before exposure. Tap water plus 0.005% DMSO (similar to the concentration of DMSO used for the imidacloprid exposures) was used as a negative control. They were subjected to the same manipulations as the fish exposed to imidacloprid. One positive mutagenic control consisting of 50 mg/L of methyl methanesulfonate was included.

Experimental design

All experiments were performed in aquaria with tap water (temperature 15 ± 1 °C, illumination with fluorescent lamps with 12:12-h light:dark periods, pH 8.5).

A bioaccumulation bioassay was designed using 5 fish per treatment. Fish were exposed individually in aquaria with 3 L of medium, and the concentration of imidacloprid in the medium (100 μg/L, 300 μg/L, and 2500 μg/L imidacloprid) was tested at 0 h, 24 h, and 48 h to check the stability. During the exposure fish were kept starved. Fish of total length 6.81 ± 0.31 cm (mean ± standard deviation [SD]) and total weight 5.85 ± 0.75 g (mean ± SD) were used. Samples of brain, muscle, gills, gut, liver, and blood were analyzed.

Two bioassays for genotoxicity were performed: 1 to test the potential genotoxicity of a commercial formulation of imidacloprid and 1 to test the individual compound. Six fish per treatment were exposed for 24 h. Concentrations of 1 μg/L, 10 μg/L, 100 μg/L, 1000 μg/L, and 10 000 μg/L imidacloprid in the commercial formulation and of 1 μg/L, 10 μg/L, 100 μg/L, and 1000 μg/L pure imidacloprid were used. Static conditions were carried out because of imidacloprid stability in aqueous solution after being tested for 48 h in the bioaccumulation bioassay. These concentrations are sublethal for *A. facetus* and were established taking into account reported environmental concentrations [15,19,21] as well as the LC50 values in model fish species available in the literature [39]. Fish of total length 9.06 ± 0.84 cm and weight 16.40 ± 4.75 g and of total length 8.64 ± 0.46 cm and weight 11.68 ± 2.29 g were selected for imidacloprid commercial formulation and imidacloprid active ingredient exposures, respectively. A detailed description of the experiments can be found below in the section *Genotoxicity tests*.

Imidacloprid analysis

Water samples were directly injected in the liquid chromatograph-tandem mass spectrometer (LC-MS/MS) to quantify the imidacloprid concentration in water.

The extraction of imidacloprid from brain (10 mg), muscle (135 mg), gills (45 mg), gut (20 mg), liver (35 mg), and blood (7 mg) was carried out following Kavvalakis et al. [40] with few modifications. Briefly, tissues were homogenized with an Ultra Turrax T8 (IKA Werke) in 2 mL methanol and incubated for 3 h in an ultrasonic bath. The bath temperature was always 38 ± 2 °C. After homogenization, the samples were centrifuged (5000 rpm, 10 min), and the supernatant was evaporated to dryness at room temperature. Only muscle samples were extracted twice. Samples were finally resuspended with 100 μL methanol and kept at –20 °C. The determination and quantification of imidacloprid were performed by LC-MS/MS (Alliance 2695 ultrahigh-performance liquid chromatograph combined with a Micromass Quattro micro™; Waters). Chromatographic separation was carried out using a precolumn SecurityGuard™ for Fusion-RP high-performance liquid chromatography columns (4 × 2.00 mm) and a Phenomenex Synergi™ Fusion RP column (50 × 2.0 mm, 2.5 μm pore). The mobile phase consisted of solution A (Milli-Q water containing 0.1 % formic acid) and solution B (acetonitrile containing 0.1% formic acid) at a flow of 0.25 mL/min. A gradient with the following conditions was performed: 0 min to 1 min 100% A, 1 min to 2 min 50% A, 2 min to 4 min A decreasing from 50% to 25%, 4 min to 4.5 min 25% A, 4.51 min 100% A maintained until 10 min. Column temperature was maintained at 25 °C with an injection volume of 10 μL. The retention time of imidacloprid was approximately 4 min.

Spectral mass data were obtained using a positive ion mode with collision energy of 20 V and electrospray ionization (ESI). Imidacloprid was detected through the transitions 256→175 mass-to-charge ratio (*m/z*) and 256→209 *m/z*. The ESI conditions used a capillary voltage of 3.5 kV, cone voltage of 20 V, source temperature of 150 °C, desolvation temperature of 300 °C, desolvation gas flow of 300 L/h, and cone gas flow of 80 L/h. The scan time was 0.5 s, with an interscan delay of 0.1 s. The limit of detection was 1 ng/mL (signal-to-noise ratio >3). The limit of quantification was 5 ng/mL (signal-to-noise ratio >5).

Genotoxicity tests

After the exposure period, blood was obtained for each fish by cardiac puncture with heparinized syringes. All fish were euthanized by transecting the spinal cord, using a razor blade. Two slides were prepared per individual by applying a drop of blood on a slide previously washed with 96% ethanol. The smears were fixed in absolute methanol for 15 min and air-dried at room temperature. Twenty-four hours after the fixation, the material was stained with 15% Giemsa solution for 15 min. To determine the micronucleus and other nuclear abnormality frequencies, 2000 erythrocytes, 1000 per slide, were analyzed from each animal under ×1000 magnification using an optical microscope (Olympus CX31). The slides were evaluated by coded and random watching. Only cells with intact cell membranes and nuclei and recognizable as erythrocytes were included. Fish erythrocytes have a well-defined rounded/ovoid nucleus. A micronucleus is characterized as a cytoplasmic nonrefractory particle, with ovoid shape and well-defined edges with the same color of the cell nucleus. To characterize the nuclear abnormality, the criteria of Carrasco et al. [41] were adopted. The criteria were divided into blebbed (nucleus with a small evagination containing chromatin), lobed (longer nucleus evagination than blebbed), and notched (nucleus with a slit without chromatin).

Calculations and statistics

Bioconcentration factors (BCFs) were calculated as the ratio of the concentration of imidacloprid in each tissue for each exposure time (micrograms per kilogram dry wt) to the mean imidacloprid concentration in water measured at the different exposure times (micrograms per liter). The Student test for normally distributed data and the Mann-Whitney test for non-normally distributed data were used for comparing different exposure times. Differences among concentrations in the accumulation assay or different treatments in the genotoxicity assay were analyzed using analysis of variance (ANOVA) or the Kruskal-Wallis test [42]. A p-value of < 0.05 was considered statistically significant.

Both micronucleus and nuclear abnormalities were calculated as number per 1000 cells. Data are expressed as average ± standard deviation. Blebbed and lobed abnormalities were grouped like "buds" (nuclear evaginations) according to Bolognesi et al. [43]. Normality and homoscedasticity of variances were verified by Shapiro-Wilk and Levene tests, respectively. To test differences among treatments, for most of the studied parameters a one-way ANOVA was applied, followed by a post hoc Dunnett test. The nonparametric Kruskal-Wallis (plus a post hoc Dunn test) test was applied in those cases where the assumption of normality or homoscedasticity of variance was not met [42]. The significance level was 5%. A linear regression analysis was conducted for testing between imidacloprid concentration and nuclear lesions

(micronucleus, total nuclear abnormalities, lobed, blebbed, notched, and bud frequencies). Spearman correlations were conducted for testing between micronucleus frequency and total nuclear abnormality, lobed, blebbed, notched, and buds [42].

RESULTS

Bioaccumulation study

The nominal and measured concentrations of imidacloprid in water at each exposure time are presented in Table 1. Water concentration of imidacloprid was 91%, 119%, and 97% of the nominal concentrations 100 μg/L, 300 μg/L, and 2500 μg/L at time 0 h; 113%, 111%, and 129% after 24 h; and 147%, 134%, and 120% after 48 h.

The concentrations of imidacloprid in brain, muscle, gills, gut, liver, and blood are shown in Figure 1. Most of the tissues showed the same pattern of concentration: the higher the time of exposure, the higher the concentration in tissues. In liver, gills, gut, and muscle the imidacloprid concentration was higher after 48 h than after 24 h, whereas in brain and blood the levels of imidacloprid were similar at both times. After 24 h and 48 h, the BCF showed partitioning to water values of approximately 1, indicating that no bioaccumulation took place in *A. facetus* (Table 1).

Imidacloprid was detected in brain under all exposure conditions (at all concentrations and times tested; Figure 1). Imidacloprid was not detected in blood of fish exposed to 100 μg/L for 24 h and 48 h but was similar at each exposure concentration when comparing between 24 h and 48 h (Figure 1).

Imidacloprid was detected in gills exposed to 100 μg/L, 300 μg/L, and 2500 μg/L at 24 h and 48 h (Figure 1). The higher the exposure concentration at the 24 h and 48 h mark, the higher the imidacloprid concentration in the gills. Furthermore, BCFs in gills were similar among the different exposure concentrations at 24 h and 48 h.

Although imidacloprid was detected in all muscle samples, muscle presented the lowest concentrations of all of the tissues tested (Figure 1). The imidacloprid concentration in muscle increased with increasing concentration in the medium.

Imidacloprid was detected in all liver samples (Figure 1). One of the highest concentrations of imidacloprid in *A. facetus* was 3.34 ± 1.02 μg/g dry weight after 48 h. The imidacloprid concentration in liver increased with increasing concentration in the medium and with time of exposure.

The highest concentration of imidacloprid (3.56 ± 0.30 μg/g dry wt) in *A. facetus* was found in the gut from fish exposed to 2500 μg/L after 48 h. The imidacloprid concentration in the gut increased with increasing concentration in the water and at 300 μg/L and 2500 μg/L imidacloprid with time.

Genotoxicity tests

The micronucleus frequency varied from 0‰ to 2‰ in the negative control samples. There were no significant differences between the negative controls from the commercial formulation and the active ingredient alone bioassays. The micronucleus frequency was significantly different between the negative and positive controls. Methyl methanesulfonate significantly increased the micronucleus frequency until 5 times the negative control (Table 2).

In the commercial formulation bioassay all fish exposed to 10 000 μg/L imidacloprid died after 3 h; therefore, genotoxicity biomarkers were not analyzed. This result indicated that the 96-h LC50 of imidacloprid for *A. facetus* is below 10 ppm. At 100 μg/L and 1000 μg/L the imidacloprid micronucleus frequency was significantly higher than its negative control (Table 2). Conversely, in the active ingredient bioassay the micronucleus frequency was significantly higher at 1000 μg/L imidacloprid in relation to its negative control (Table 2 and Figure 2).

The linear regression between imidacloprid concentration and micronucleus frequency was positive and significant when tested using the commercial formulation as well as the active ingredient alone ($R^2 = 0.27$, $p < 0.05$; $R^2 = 0.61$, $p < 0.0001$, respectively; Figure 3A,B).

There were no significant differences in total nuclear abnormality or any individual nuclear abnormality frequencies between the negative controls from the 2 bioassays. The positive control (methyl methanesulfonate) increased the total nuclear abnormality frequency 2-fold in relation to the negative control, although this difference was not significant (Table 3). In the commercial formulation bioassay, at 10 μg/L imidacloprid, significant differences were found for blebbed abnormalities in comparison to the negative control (Figure 2), whereas in the active ingredient bioassay lobed abnormalities showed increases at 1000 μg/L imidacloprid (Table 3). The linear regression analysis revealed significant coefficients between imidacloprid concentration and lobed nuclei as well as between imidacloprid concentration and buds (Figure 3C,D). The remaining nuclear abnormality showed no significant

Table 1. Nominal and measured concentrations of imidacloprid in water at the beginning of the experiment (0 h) and after 24-h and 48-h exposures and bioconcentration factors in tissues of *Australoheros facetus* exposed to 100 μg/L, 300 μg/L, and 2500 μg/L at 24 h and 48 h

	Imidacloprid concentration (μg/L) in water[a]		Bioconcentration factor per tissue (L/kg)[b]					
Time	Nominal	Measured	Brain	Blood	Gills	Muscle	Gut	Liver
0 h	100	91.2 ± 17.6						
24 h		113.2 ± 16.5	1.4 ± 0.2	n.c.	0.3 ± 0.2	0.2 ± 0.1	n.c.	0.5 ± 0.1
48 h		147.5 ± 33.0	1.8 ± 0.7	n.c.	0.4 ± 0.1	0.6 ± 0.1	1.2 ± 0.2	1.5 ± 0.6
0 h	300	357.5 ± 4.6						
24 h		336.9 ± 104.4	1.0 ± 0.3	0.9 ± 0.5	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.1	0.5 ± 0.2
48 h		403.2 ± 17.6	0.9 ± 0.3	0.6 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	1.3 ± 0.3	1.4 ± 0.4
0 h	2500	2414.3 ± 327.5						
24 h		3226.3 ± 399.0	0.7 ± 0.5	0.4 ± 0.2	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.4 ± 0.3
48 h		3011.7 ± 216.3	0.9 ± 0.4	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	1.0 ± 0.6	1.2 ± 0.4

[a]Data of imidacloprid concentration in water are expressed as mean ± standard deviation ($n = 3$).
[b]Bioconcentration factors are expressed as mean ± standard deviation ($n = 5$).
n.c. = not calculated, values under the limit of quantification.

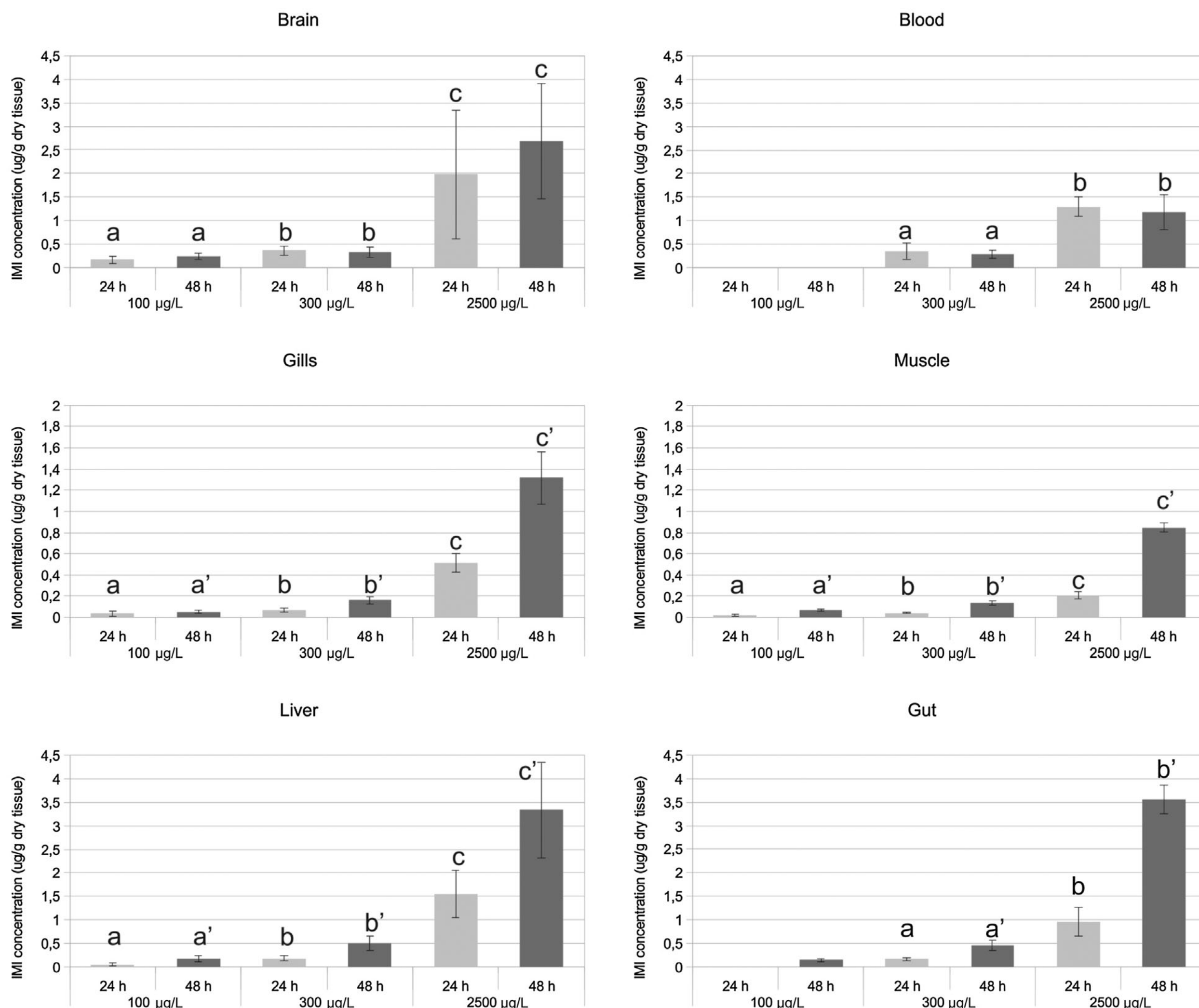

Figure 1. Imidacloprid concentrations in brain, blood, gills, muscle, liver, and gut of *Australoheros facetus* fish exposed to 100 μg/L, 300 μg/L, and 2500 μg/L at 24 h and 48 h. Different letters indicate significant differences among exposure concentrations at the same time. a versus a', b versus b', and c versus c' indicate significant differences between times at the same exposure concentration. IMI = imidacloprid.

relationships ($p > 0.05$). All correlations attempted between the micronucleus frequencies and the frequencies of the 3 nuclear lesions, together with total nuclear abnormality or individually, were not significant (Table 4).

Table 2. Micronucleus frequency expressed as mean ± standard deviation in erythrocytes of the freshwater fish *Australoheros facetus* ($n = 6$) exposed to imidacloprid

Chemicals	Concentration (mg/L)	No. of cells analyzed	Micronucleus (‰)
MMS (Co+)		10 324	4.65 ± 1.41*
DMSO (Co−)		10 095	0.89 ± 0.42
Imidacloprid	1	10 078	0.89 ± 0.41
(commercial	10	12 141	1.07 ± 0.80
formulation)	100	12 127	2.39 ± 0.84*
	1000	11 840	3.58 ± 0.83*
DMSO (Co−)		12 000	0.92 ± 0.74
Imidacloprid	1	11 000	1.33 ± 0.93
(active ingredient)	10	10 650	1.42 ± 0.74
	100	11 000	1.92 ± 0.66
	1000	12 000	2.58 ± 0.97*

*Significant difference from control (Co−).
Co− = negative control; Co+ = positive control; DMSO = dimethyl sulfoxide (0.005%); MMS = methyl methanesulfonate (50 mg/L).

DISCUSSION

Bioaccumulation study

The extraction method used showed effectiveness for quantification of imidacloprid from different tissues at all concentrations tested with the exception of blood at the lowest concentration tested (100 μg/L imidacloprid). The small increase in the imidacloprid concentration with exposure time observed (percentages from nominal concentrations higher than 100) might be the product of water evaporation.

Persistent and bioaccumulative organic chemicals have been conventionally monitored over many years, but currently less persistent and less hydrophobic organic compounds are used as pesticides. Despite their lesser bioconcentration potential, relatively large fluxes of some of these compounds into aquatic systems might be acutely toxic and/or induce sublethal chronic abnormalities [44]. In *A. facetus* BCFs for imidacloprid were low, given its hydrophilic character ($\log K_{OW} = 0.57$ [38]). No bioaccumulation other than that in the brain tissue at 100 μg/L imidacloprid was observed; in this case a BCF higher than 1 was obtained because of the high affinity of the insecticide for the nicotinic receptors of the nervous system. Although it is unlikely that an equilibrium condition between incorporation

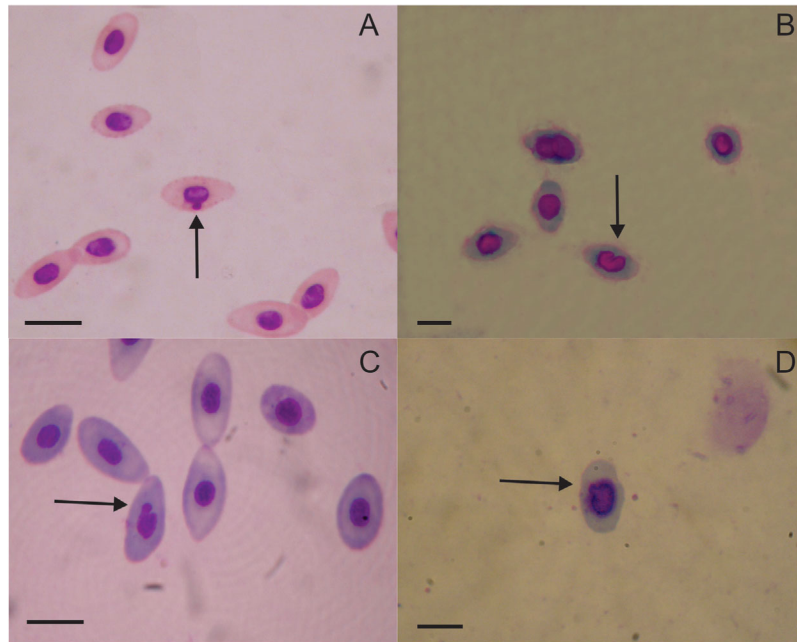

Figure 2. Photomicrographs of peripheral erythrocytes from *Australoheros facetus* exposed to imidacloprid. (**A**) Micronucleus, (**B**) notched nuclei, (**C**) lobed nuclei, (**D**) blebbed nuclei. Giemsa-stained blood smear. Bar = 10 μm.

and elimination of imidacloprid can be reached after only 48 h, BCFs are helpful not only to test if the fish tissues were able to accumulate high levels of imidacloprid from the water but also to understand if the imidacloprid concentration in the medium affects the uptake rate of imidacloprid into the fish. Because the concentrations used in the bioaccumulation bioassay were higher compared with the residue levels commonly found in surface waters, the BCFs obtained (mostly lower than 1) help us to understand that in the natural environment fish are not going to have residues in their tissues higher than those found in the waters in which they live. However, using commercial formulations higher accumulation rates compared to the values observed in the present study using only the active ingredient can be expected. This fact could be expected because of the presence of coformulants (e.g., DMSO, methylpyrrolidone), which can modify its bioavailability [35].

The fact that imidacloprid was found in brain tissue, independent of the concentration in the exposure medium, indicates that imidacloprid was overpassing the brain–blood barrier, which is an essential structure to maintain brain homeostasis and to protect the organ from toxic substances [45]. In general, the more hydrophilic the compound, the less likely it will cross the brain–blood barrier; however, some hydrophilic compounds may gain access by piggybacking on specific transporters that are present in this barrier [23]. The observed imidacloprid occurrence in brain tissue yields to the

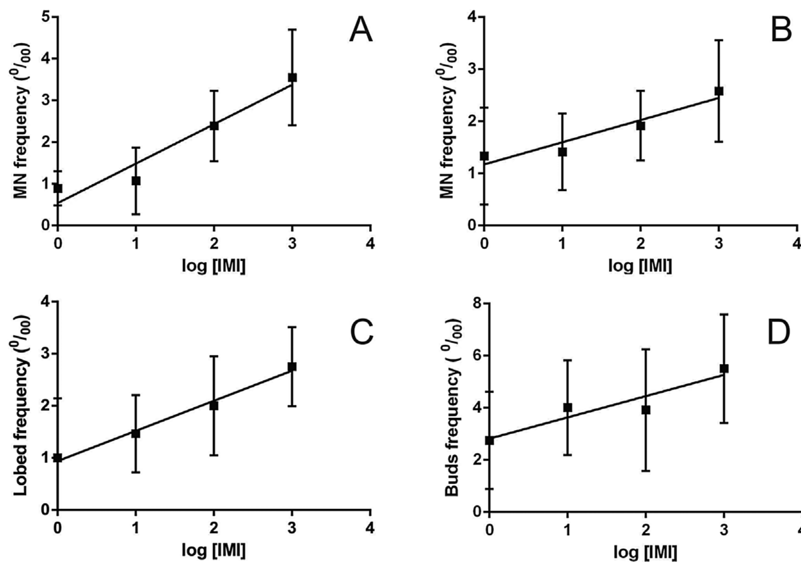

Figure 3. Linear regression between imidacloprid concentration and genetic biomarkers in the freshwater fish *Australoheros facetus* exposed to imidacloprid. (**A**) Imidacloprid commercial formulation, micronucleus frequency. (**B**) Imidacloprid active ingredient, micronucleus frequency. (**C**) Imidacloprid active ingredient, lobed frequency. (**D**) Imidacloprid active ingredient, bud frequency. IMI = imidacloprid; MN = micronucleus.

Table 3. Nuclear abnormality frequency expressed as mean ± standard deviation in erythrocytes of the freshwater fish *Australoheros facetus* ($n = 6$) exposed to imidacloprid

		Nuclear abnormality frequency (‰)			
Chemicals	Concentration (μg/L)	Lobed	Notched	Blebbed	Total
MMS (Co$^+$)		3.60 ± 1.38	9.48 ± 4.15*	0.67 ± 1.50	13.76 ± 5.75
DMSO (Co$^-$)		3.10 ± 1.54	3.37 ± 1.75	1.06 ± 1.09	7.53 ± 2.69
Imidacloprid (commercial formulation)	1	1.60 ± 1.45	3.58 ± 2.18	4.00 ± 1.84	9.18 ± 4.29
	10	2.06 ± 1.53	6.12 ± 4.95	4.77 ± 3.13*	12.95 ± 8.87
	100	4.94 ± 2.11	6.90 ± 3.45	3.62 ± 0.98	15.47 ± 5.54
	1000	2.52 ± 1.16	3.95 ± 1.00	2.18 ± 1.21	8.65 ± 2.05
DMSO (Co$^-$)		1.00 ± 0,55	3.33 ± 1.29	2.00 ± 0.89	6.33 ± 2.14
Imidacloprid (active ingredient)	1	1.00 ± 1.14	2.92 ± 1.74	1.75 ± 1.21	5.92 ± 3.37
	10	2.06 ± 1.59	3.46 ± 1.69	1.95 ± 0.51	7.47 ± 2.78
	100	2.00 ± 0.95	3.67 ± 2.75	1.92 ± 1.93	7.83 ± 4.87
	1000	2.75 ± 0.76*	4.83 ± 2.89	2.75 ± 1.44	10.42 ± 5.05

*Significant difference from control (Co$^-$).
Co$^-$ = negative control; Co+ = positive control; DMSO = dimethyl sulfoxide (0.005%); MMS = methyl methanesulfonate (50 mg/L).

presumption of possible negative effects in the central nervous system. Moreover, the brain has a low defense level against toxic effects produced by oxidative stress [46,47]. Hence, special attention to brain tissue is recommended for further studies of toxic effects from neonicotinoid exposure. The higher imidacloprid concentration in the brain is most likely the result of its strong affinity for the nicotinic receptors on the neuronal membranes, to which imidacloprid binds irreversibly, independently of the lipophilicity of this compound. Imidacloprid's main effect in brain tissue is related to its specific mode of action as an agonist neurotoxicant, by activating the nicotinic receptors, which is poorly known in vertebrates and more particularly in fish. In humans, impaired short-term memory and other neurological illnesses such as "accommodation disorder" and "finger tremor" have been associated with neonicotinoids [48].

Similar to brain tissue, the increase of imidacloprid concentration in blood was not directly proportional to the increase of imidacloprid concentration in the exposure medium. Once a toxicant crosses a cell membrane, the principal distribution mechanism is through the blood circulatory system [49]. According to our results, imidacloprid is immediately distributed to other tissues via the circulatory system, but it is also able to exert sublethal effects such as genotoxicity. Although there are no other available data concerning the effects of neonicotinoids in fish blood, other kinds of pesticides like organophosphates [50] and organochlorines [51] have been reported to negatively affect such blood cells as erythrocytes and leukocytes as well as affecting hemoglobin levels.

Table 4. Correlation between micronucleus (‰) and nuclear abnormality (total, lobed, blebbed, notched, and buds; ‰) frequencies in the freshwater fish *Australoheros facetus* exposed to imidacloprid

Treatment	Correlation	*p*-value	R^a
Imidacloprid (commercial formulation)	MN vs total NA	0.227	0.262
	MN vs lobed	0.308	–0.222
	MN vs blebbed	0.186	0.285
	MN vs notched	0.09	0.112
	MN vs buds	0.882	–0.032
Imidacloprid (active ingredient)	MN vs total NA	0.523	0.137
	MN vs lobed	0.905	–0.025
	MN vs blebbed	0.695	0.084
	MN vs notched	0.621	0.106
	MN vs buds	0.789	0.057

[a]Spearman correlation coefficient.
MN = micronucleus; NA = nuclear abnormality.

The BCF in gills was not modified by the imidacloprid concentration in the exposure medium. Because water-soluble chemicals (e.g., imidacloprid) dissolve readily in water, they are easily available to the gill epithelium [52]. The combination of countercurrent exchange between water and blood, large surface area (secondary lamellae), and short diffusion distance separating water and blood results in highly efficient extraction of the small concentrations of oxygen present in water [23] and probably of imidacloprid because the absorption of imidacloprid in gills seemed to occur rapidly. Because gills are the main site for gas exchange, hydromineral regulation, and nitrogenous excretion, possible physiological effects in the functionality of gills from imidacloprid exposure can be expected. Particularly, a stress syndrome in fish exposed to imidacloprid coupled with a parasitic infection has been reported [30].

Several primary factors including the lipid solubility of the compound control the distribution of xenobiotics from blood to peripheral tissues [23]. Because of the hydrophilic nature of imidacloprid, we hypothesize that the main way that it enters muscle would be from the blood supply rather than the skin. Because muscle is the edible tissue of fish, the detection of imidacloprid in this tissue after only 24 h of exposure highlights the importance of evaluating it.

Although fish were starved during the exposure, the digestive pathway seemed to be the most important destination for imidacloprid in the body because gut tissue showed the highest concentrations of the insecticide. In fact, the intestine is 1 of the main targets of dietary and waterborne toxic agents, and given the important osmoregulatory function of the intestine, it directly affects the regulation of water and ion balance [23]. Although most toxicological studies deal with the effect of waterborne pollutants on freshwater fish, which have low drinking rates, the present results demonstrate that it should always be evaluated. In the present study, the presence of imidacloprid in the gut might be a result of the fact that fish drank the exposure solution in addition to the presence of nicotinic acetylcholine receptors in the gastrointestinal tract [48]. Given the liver complement of enzymes, its dual blood supply, and the enterohepatic circulation, this organ is a major target for xenobiotics whatever the exposure route

considered [23]. Indeed, liver of *A. facetus* was also an important target organ for imidacloprid, presenting a proportional relationship between the concentrations in the medium and in the organ. In addition, imidacloprid concentration in liver increased by a factor >2 from 24 h to 48 h of exposure. Although there are no data concerning the effects of imidacloprid in fish liver, oxidative stress and inflammatory processes have been reported in liver of rats [53,54]. Based on the present data and the fact that the damage of hepatic structure and function not only is relevant for the organ itself but may also affect other organs, future work would be focused on detecting possible negative effects in liver.

Genotoxicity tests

The micronucleus frequencies (0–2‰) found in negative controls are similar to those obtained by Crupkin et al. [27] with endosulfan in the same species (1.7–3.7‰). Bolognesi et al. [43] reported the spontaneous frequency of micronucleated erythrocytes in fish from different species, finding that it ranged between 0‰ and 1.75‰. Therefore, the frequencies found in *A. facetus* could be considered appropriate for experimentation. The sensitivity and reproducible responses of the species could enable the detection of hazards in the aquatic ecosystems arising from agricultural, domestic, and industrial contamination. The frequency of micronucleus in *A. facetus* was not significantly different between negative controls from the 2 bioassays denoting the reproducible response in *A. facetus*.

No previous data on the genotoxicity of imidacloprid to fish are available. Micronuclei analysis demonstrated that imidacloprid was able to damage the DNA of *A. facetus* at 100 μg/L, a concentration 1 order of magnitude higher than that found to be genotoxic in amphibians like *Rana* sp. (50 μg/L) [35]. All other genetic biomarkers of toxicity in vertebrates were evaluated in the order of milligrams per liter [35–37]. For example, genotoxic effects have been reported in amphibians at 8000 μg/L to 32 000 μg/L imidacloprid [35] and at 25 000 μg/L commercial formulation Glacoxan Imida [37]. The concentrations of 1 μg/L and 10 μg/L tested in the present study, which are high environmentally relevant concentrations, did not show significant micronucleus frequency increment. Therefore, it is important to note that the effect observed in the present study as well as in amphibians corresponds to concentrations higher than those more commonly found in the environment.

The regression analysis between micronucleus and imidacloprid concentration indicated a significant concentration-dependent increase of genotoxicity. Exposures with the active ingredient alone showed an incremented micronucleus frequency at 1000 μg/L, whereas fish exposed to the commercial formulation showed increased micronucleus at 100 μg/L and 1000 μg/L, indicating the higher potency of the product IMIDA NOVA 35 tested. This is in agreement with other toxicity data; for example, when imidacloprid was formulated as Confidor SL 200, it was more toxic to adult zebrafish than analytical-grade imidacloprid [39]. It is noteworthy that the inert ingredients in the commercial formulation used in the present study are unknown, but for other trademarks of commercial imidacloprid, like Evidence® or Confidor® (Bayer), the presence of additives like DMSO, methylpyrrolidone, propylenecarbonate, and mineral oil has been described. Adjuvants constitute a broad range of substances, of which solvents and surfactants are the major types. They are used to enhance adsorption, penetration, and translocation of the active ingredients into the organisms [55]. Therefore, they can enhance the absorption of imidacloprid by cell membranes but also might be responsible for the genotoxic effect observed.

Micronucleus can have 2 different origins: the disruption of the mitotic spindle (aneunogenic) and the fragmentation of DNA (clastogenic). The micronucleus of *A. facetus* exposed to IMIDA NOVA 35 was size-classified according to Hashimoto et al. [56] into type I (small) or type II (large; data not shown). According to this classification, small micronuclei are induced by clastogens and large micronuclei are preferentially induced by spindle poisons. Our results showed a predominance of small micronucleus at 100 μg/L and 1000 μg/L, indicating a clastogenic origin of micronucleus caused by this insecticide. This corresponds with the capacity of imidacloprid to induce DNA damage in bone marrow cells of rats, measured by micronucleus and chromosome aberrations (chromosome breaks and stickiness) [57] and in human hepatoma cells (HepG2) in which 76.6% of micronuclei did not present a centromeric signal [58].

In agreement with the micronucleus, lobed nuclei and buds also showed a significant dose–effect relationship with imidacloprid concentration, supporting the idea that these nuclear abnormalities could be biomarkers of genotoxicity. Bolognesi et al. [43] found a correlation between micronucleus frequency and nuclear evaginations or buds and recognized the importance of recording this anomaly to improve the information obtained with the micronucleus assay. In the present study no correlation was found, indicating that nuclear abnormality and micronucleus may not have a common origin. Indeed, the use of a recognized genotoxic compound in the positive control did not show significant changes in total nuclear abnormality with respect to the negative controls. Recent literature demonstrates a controversy about this relationship. Although Carrasco et al. [41] found that micronuclei correlated with both blebbed and lobed nuclei, Ayllon and Garcia-Vazquez [59] found induction of nuclear abnormalities even if micronuclei were not induced. Although its genotoxic origin is still not clear, lobed frequency has been used as a genotoxic parameter, being related to oxidative DNA lesions in a positive and significant way [60]. Indeed, Oliveira et al. [60] concluded that the positive correlation between 8-hydroxy-2-deoxyguanosine and nuclear abnormalities suggests oxidative stress as a mechanism involved in the formation of nuclear abnormalities.

Genetic alterations in somatic tissues can have a number of immediate effects on the cells involved, including death or transformation into malignancy. Beginning at the molecular level, mutagens interact with DNA to form lesions (somatic effect) that can cause cell or tissue damage, which can lead to adverse health effects or stress on the individual. Consequently, this can lead to reproductive impairment or high mortality rates, which in turn can cause population bottlenecks (an ecological effect) and finally result in the reduction of genetic variability in populations (population genetic effects) [32]. The results of the tests carried out with the ingredient imidacloprid and the commercial formulation in *A. facetus* cells indicated that genotoxic effects are present at high environmentally relevant concentrations on the order of micrograms per liter. Therefore, it should be highlighted that the substantial use of imidacloprid in the world—to date 1 of the most sold insecticides—could result in increasing concentrations of it in the environment and consequences for freshwater ecosystems in the near future.

The present study is the first study of imidacloprid uptake and tissue distribution in aquatic vertebrates. Imidacloprid showed a high stability in water, which is an important factor for

possible acute effects to aquatic biota. It reached all the tissues studied such as blood, liver, gut, brain, muscle, and gills. Our results highlight the importance of studying different target tissues of imidacloprid in fish and potential sublethal effects in some of them. In the present study genotoxicity was detected and showed a dose-dependent occurrence, but further studies are necessary to explore potential adverse effects in other tissues. Although imidacloprid is highly soluble in water, it was not bioconcentrated; because concentrations in tissues were on a par with those in the medium, it was able to cause relevant genotoxic effects in fish.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (mirta.menone@gmail.com).

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